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Fed-batch operational strategies for recombinant Fab production with *Pichia pastoris* using the constitutive *GAP* promoter

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ABSTRACT

Carbon source and growth rate are two major parameters affecting recombinant protein production in *Pichia pastoris*. The effect of the most commonly used carbon sources (glycerol or glucose) and the specific growth rate (μ) has been studied on the production of a human antigen-binding fragment (Fab) in this cell factory under the constitutive GAP promoter in fed-batch cultures.

Glycerol for batch phase and glucose for fed-batch phase was the most successful carbon source combination. During batch phase, by-products were detected when glucose was used, despite maintaining DO at values higher than 35%. Also, the presence of cell aggregates was detected affecting the reproducibility and operability of the bioprocess.

Conversely, glucose was the best substrate for fed-batch phase. When working at C-limiting conditions, neither by-products nor aggregates were detected and Fab production levels were comparable to those obtained with glycerol. In addition, the lower heat yield ($Y_{Q/X}$) and oxygen to biomass yield ($Y_{O_2/X}$) for glucose-supported cultures made this substrate the best alternative from an industrial operational point of view.

In addition, the effect of specific growth rate on fed-batch Fab production was studied. Medium and high μ (0.10 and 0.15 h^{-1}) set-points showed similar Fab production yield. However, in terms of total and volumetric productivity, higher μ was the best process condition.

1. Introduction

Recombinant proteins, including biopharmaceuticals proteins and industrial enzymes, is a multi-billion dollar market [1,2]. *Pichia pastoris* is currently one of the most effective and versatile systems for the expression of heterologous proteins [3]. This fact is due to the combination of traits of this yeast, such as its accessibility for genetic manipulation including well characterized genetic elements, the ability to grow on defined media at very high-cell densities, its capacity to perform post-translational modifications including glycosylation and disulphide bond formation and the possibility of driving the protein production intracellularly or secreting it to the extracellular medium [4–6].

Different promoters have been successfully employed for recombinant protein production in *P. pastoris* [7]. The alcohol oxidase promoter (P_{AOX1}), which is strongly induced in the presence of methanol, has been extensively used, obtaining very high expression levels [8–10]. Nevertheless, during the last years, the glyceraldehyde-3-phosphate dehydrogenase constitutive promoter (P_{GAP}) has become an increasingly used alternative [11–13]. *GAP* promoter allows the protein expression using glucose, glycerol and other carbon sources as a substrate [14]. Several studies have reported that P_{GAP} is more efficient than P_{AOX1} [15–17], whereas others showed opposite results [18,19]. Thus, it appears that expression levels achieved for a given protein using different promoters vary significantly based on properties of the expressed protein. Additional research would be required to determine which factors impact the efficiency of both promoters [3]. The use of the constitutive *GAP* promoter avoids the use of methanol in the fermentations, which reduces the cell lysis of the cultures, and subsequently, the proteolysis of secreted proteins [20]. From a large-scale processing perspective, the P_{GAP} expression system may be advantageous because it eliminates the hazard and cost associated with the storage and delivery of large volumes of methanol [21] and significantly decreases heat production and oxygen requirements of the processes [22].

Full-size monoclonal antibodies (mAbs) and their fragments are an increasingly class of therapeutic agents. Main characteristics and applicability have been widely described previously [23,24]. Antigen-binding fragments (Fab) are complex proteins composed of the antigen binding regions of an antibody molecule, containing both the heavy chain domains vH–cH and the entire light chain

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Nomenclature

List of symbols

CPR	carbon dioxide production rate (mol CO_2 L^{-1} h^{-1}).
F_i	volumetric feeding rates of the components (L h^{-1})
OUR	oxygen uptake rate (mol O_2 L^{-1} h^{-1}).
P	product concentration (mg Fab L^{-1})
Q_P	volumetric productivity (mg Fab L^{-1} h^{-1})
q_P	specific product formation rate (mg Fab g_X^{-1} h^{-1})
q_S	specific substrate uptake rate (g_S g_X^{-1} h^{-1})
S	substrate concentration (g L^{-1})
V_i	volume (L)
X	dry biomass concentration (g L^{-1})
$Y_{CO_2/X}$	carbon dioxide to biomass yield coefficient (mol CO_2 g_X^{-1})
$Y_{O_2/X}$	oxygen to biomass yield coefficient (mol O_2 g_X^{-1})
$Y_{P/S}$	product to substrate yield coefficient (mg Fab g_S^{-1})
$Y_{P/X}$	product to biomass yield coefficient (mg Fab g_X^{-1})
$Y_{Q/X}$	heat yield coefficient (kJ g_X^{-1})
$Y_{X/S}$	biomass to substrate yield coefficient (g_X g_S^{-1})

Greek symbols

μ	specific growth rate (h^{-1})
ρ_i	density (g L^{-1})
σ	fraction of dry matter in the biomass (g g^{-1})
ΔH_i	combustion enthalpy of the components (kJ g^{-1})

vL–cL chains, which are connected via disulfide bonds [25]. Microbial expressions systems have been investigated for their potential to produce mAbs and different mAbs fragments. The major advantages of these systems lie in their shorter process times and lower production costs as compared to mammalian cell culture [26]. In *P. pastoris*, high expression levels of mAbs and their fragments have been already achieved [27,28].

In this study, the human Fab 2F5 was used as a model protein complex in order to evaluate the effect of the carbon source and the specific growth rate for the production of heterologous protein under the control of the glycolytic *GAP* promoter. Glucose and glycerol were selected and compared as alternative substrates in both phases of the process, batch and fed-batch. Once the best combination of substrates was selected, three different specific growth rates were evaluated and compared. Thorough studies concerning specific rates including cell growth, substrate consumption and production formation have been carried out.

2. Materials and methods

2.1. Strains

The *P. pastoris* strain X-33 pGAPZαA Fab2F5 expressing the human 2F5 Fab under the control of *P. pastoris* constitutive *GAP* promoter was used throughout this study. Briefly, the expression cassettes for the light and heavy chain of the human Fab 2F5 antibody fragment were separately introduced under the control of the *P. pastoris GAP* promoter and combined on one plasmid [25]. This strain expresses the human Fab using the *Saccharomyces cerevisiae* α-mating signal sequence for secretion of the heterologous protein to the extracellular medium.

2.2. Cultivation methods

2.2.1. Inoculum preparation

The inoculum for bioreactor cultures were grown for 24 h in 1 L baffled shake flasks at 25 °C, 130 rpm, in YPG medium (2% peptone, 1% yeast extract, 2% glycerol; pH=7) adding zeocin (100 μg mL^{-1}). The shake flasks containing 150 mL of culture medium were inoculated from cryostocks of the recombinant strain, grown, harvested by centrifugation and re-suspended in batch medium to be inoculated into the bioreactor.

2.2.2. Fed-batch cultivation

Fed-batch cultivations were carried out in a 5 L Biostat B Bioreactor (Braun Biotech, Melsungen, Germany) working at initial volume of 2 L and finishing the process at approximately 4 L. The culture conditions were monitored and controlled at the following values: temperature, 25 °C; pH, 5.0 with addition of 30% (v/v) ammonium hydroxide; pO_2, above 20% saturation by controlling the stirring speed between 600 and 1200 rpm and using mixtures of air and O_2 at aeration within 1.0 and 1.25 vvm. Water evaporation losses were minimized during the processes using an exhaust gas condenser with cooling water at 8 °C.

The standard fermentation strategy consisted of two phases. First, the batch phase starts with a standard carbon source concentration (40 g L^{-1}). It was performed using the batch medium described elsewhere [11]. Glycerol and glucose were alternatively used as sole carbon sources in different fermentations. During this step, yeast grows at maximum specific growth rate until the depletion of the C-substrate achieving a moderate concentration of biomass (≈20 g L^{-1}). Just after that, begins the fed-batch phase, the most important of the process, where the culture reaches high concentrations of biomass and product.

Second, the fed-batch phase was carried out by adding feeding medium, which have similar composition to that described by Maurer et al. [11] with minor modifications detailed below. Glycerol and glucose were alternatively used as carbon source (400 g L^{-1}). Biotin 0.02% (6 mL), PTM_1 (15 mL) trace salts stock solution [29] and antifoam Struktol J650 (0.2 mL) were added per litre of feeding medium.

The fermentation strategy during the fed-batch phase aimed to achieve pseudo-steady-state conditions for specific rates during carbon-limiting growth. A pre-programmed exponential feeding rate profile for substrate addition derived from mass balance equations to maintain a constant specific growth rate (μ) was implemented [30]. This open-loop control structure allows maintaining a constant μ, which enhances process reproducibility and facilitates the systematic study of growth rate-related effects on heterologous protein production [3].

The equations derived from the fed-batch substrate balance used to apply this strategy were described elsewhere [31,32]. A constant biomass to substrate yield ($Y_{X/S}$ = 0.5 g_X g_S^{-1}) was considered to determine the initial feed rate. The feeding medium was added by automatic Crison micro-burettes MicoBU-2031 (Alella, Barcelona, Spain).

2.3. Analytical methods

2.3.1. Biomass determination by dry cell weight (DCW)

Biomass concentration of the samples was determined as DCW using the method previously described [31]. Determinations were performed by triplicate and the relative standard deviation (RSD) was about 4%.

2.3.2. Product quantification

The 2F5 human Fab was quantified by ELISA as previously described [33]. Determinations were performed by triplicate and the RSD was about 4%.

2.3.3. Protein quantification

Extracellular protein concentration was determined with the bicinchoninic acid protein assay kit (Pierce BCA Protein Assay, Prod.